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An Improved Quantitative Assay for Chemokinesis in *Tetrahymena*

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Abstract. This paper presents a quantitative and sensitive assay for the measurement of chemosensory behavior in *Tetrahymena.* The two-phase assay is easy to perform in large quantities, so a variety of compounds can be screened under comparable conditions. A suspension of 2×10^5 cells ml^{-1} (the upper phase) is starved for 20–40 h and then gently placed on top of a 5% solution of Metrizamide (the lower phase) in a disposable microcuvette. The optical density of the lower phase is monitored at 600 nm with an automated spectrophotometer at selected time points. Optimum sensitivity of the assay is achieved when the cells slowly but continuously enter the lower phase, so that about 5% of them will be in the lower phase within 30 min. Optimal chemosensory responses occurred in *Tetrahymena thermophila* at about 25°C. The response was delayed at 15°C and markedly reduced at 35°C. The data suggest three bases for quantifying the response in the assay: (1) initial slope of the absorbance *versus* time; (2) final maximal absorbance within the time period of measurement; and (3) signal-to-noise ratio (S/N) at a fixed time. We have quantified—in terms of S/N—the chemosensory responses in *Tetrahymena* for the following compounds: β-endorphin, fibroblast growth factor, insulin, and platelet-derived growth factor (PDGF); these substances were active in nanomolar concentrations, and the maximal S/N was between 3 and 5.1. Acetylcholine was active only in millimolar concentrations; maximal S/N was 4.1 at 1 m*M*. Glutamic acid, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine were active in millimolar concentrations, with S/N between 1.5 and 2.5. We found S/N equal to 9.6 for a mixture of amino acids (1 m*M* of each amino acid). Furthermore, if PDGF was added to the mixture of amino acids, the S/N increased to 11.3. Proteose peptone at a concentration of 1 mg ml^{-1} produced a strong response, with S/N equal to 17.5. The assay is also suitable for the detection and quantification of repellents. We found that 20 m*M* KCl, 1 m*M* 4-nitroaniline (4-NA), and 100 μ*M* 2,4-dinitroaniline (2,4 DNA) each almost completely prevented the cells from migrating into the lower phase. The minimum concentrations of significant repellent effect using these substances were 500 μ*M*, 1 μ*M*, and 50 n*M*, respectively.

Introduction

Several assays have been proposed for the measurement of chemokinesis in *Tetrahymena,* including capillary assays (Almagor *et al.,* 1981; Leick and Helle, 1983; Levandowsky *et al.,* 1984), modified Zigmond chambers (Leick, 1988; Hellung-Larsen *et al.,* 1990), field assays (Hellung-Larsen *et al.,* 1986), and a membrane filter assay (Leick *et al.,* 1990). A common feature has been the time-consuming and labor-intensive setup of the assays, involving large volumes of cell suspension and microscopical or electronic counting of the cells. None of the assays have been useful for testing more than a few samples at a time, so precise and direct comparisons of many samples at the same time have been difficult to perform. Because of its chemosensory sensitivity to certain anilines and phenols, *Tetrahymena* has been proposed as a test organism in the screening of industrial aquatic pollutants (Berk *et al.,* 1990; Pauli and Berger, 1992). Therefore, a sensitive and easy-to-perform assay for chemokinesis in *Tetrahymena* will increase the usefulness of this organism in the area of aquatic ecotoxicology.

To make a detailed examination and comparison of the kinetics of the chemosensory response of *Tetrahymena*

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at different experimental conditions, an assay that produces precise time-response curves must be established. Indeed, none of the existing assays yielded more than a few points of the time-response curve of the chemosensory response of *Tetrahymena*.

Recently we found that control of the physiological state of the cells improved the reproducibility and sensitivity of a capillary assay for chemokinesis in *Tetrahymena* (Koppelhus *et al.*, 1994). Encouraged by these results, we have reinvestigated the two-phase assay that was introduced in 1986 as a semiquantitative method (Hellung-Larsen *et al.*, 1986). A quantitative assay with high sensitivity was then developed as described here.

Materials and Methods

Cell cultures

Tetrahymena thermophila strain B7 was grown axenically in PY medium: 0.75% proteose peptone (Difco), 0.75% yeast extract (Difco), 1.5% glucose, 1 m*M* $MgSO_4$, 50 μM $CaCl_2$, and 100 μM ferric citrate. The PY medium was diluted with water to ⅓ prior to use. Fifty-milliliter cultures of B7 cells were grown at 35°C in 500-ml Fernbach flasks. The cultures were left unagitated to avoid cell division stress (Hellung-Larsen and Lyhne, 1992). Cells in exponential growth phase were used to inoculate the cultures to a concentration of about 10^4 cells ml^{-1}.

Transfer to starvation was done after 16–20 h at a cell density of about 8×10^5 cells ml^{-1} (late exponential growth phase) or 40–50 h at a cell density of approximately 1.3×10^6 cells ml^{-1} (early stationary phase). For starvation, the cells were collected by centrifugation at 500 × *g* for 3 min and then gently resuspended in deionized water to a cell concentration of approximately 1.5×10^5 cells ml^{-1}. The cells were starved at 21°C in 500-ml Fernbach flasks containing, at a maximum, 50 ml of cell suspension each. This ensured a constant maximal oxygen saturation of the suspensions of starving cells (Koppelhus *et al.*, 1994).

Cell concentration and cell volumes were determined with the aid of a Coulter Multisizer, as previously described (Hellung-Larsen and Andersen, 1989).

Chemoattraction assays

Capillary assay. A capillary technique with single glass capillaries was used. Heparinized glass capillaries (75 mm long, inner diameter 1.1–1.2 mm) were filled with test solution, and one end of the capillary was sealed with wax. The capillary was then placed horizontally through a sealed hole into the jar containing the cell suspension, and the open end of the capillary was brought in contact with the cell suspension. Incubation was usually for 45 min at 28°C. After incubation, the number of cells accumulated in the capillary was determined by microscopical counting in a hemacytometer. All experiments were carried out in series of five identical assays and five parallel control assays.

Two-phase assay. The chemoattractant was dissolved in 2–5% (w/v) metrizamide (2-[3-acetamido-5-N-methyl-acetamido-2,4,6-triiodo benzamido]-2-deoxyglucose) in deionized water and 1 ml was placed—as the lower phase—in a disposable plastic cuvette (Plastibrand, Germany, Cat. No. 759015). A 1.5-ml suspension of starved cells was then carefully layered on top of the metrizamide solution and the optical density (OD_{600}) of the metrizamide phase was monitored automatically every 2 min in a thermostated recording spectrophotometer (Shimadzu UV-160), where six cuvettes could be monitored in parallel. Assays were performed at 28°C, unless otherwise stated. The assay procedure is shown schematically in Figure 1.

The response at a given time was calculated as

$$\frac{OD_{600} \text{ of the lower phase}}{1.5 \times (OD_{600} \text{ of the original cell suspension used})} \times 100\%$$

The 100% response corresponds to the theoretical situation in which all cells from the upper phase (1.5 ml original cell suspension) are equally distributed throughout the lower phase (1 ml of metrizamide), thus giving rise to an optical density 1.5 times that of the cell suspension.

We found that the OD_{600} of an aqueous cell suspension is linearly proportional to the concentration of cells, when cells are equally distributed throughout the suspension (Figure 2). In the assay, we found that cells—when present in the lower phase—in fact tend to be equally distributed within the phase. This is in accordance with the observations reported earlier (Hellung-Larsen *et al.*, 1986). The OD_{600} of 5% metrizamide was equal to the OD_{600} of water.

Chemicals

Acetylcholine, amino acids, bovine serum albumin (BSA), β-endorphin, met-enkephalin, fibroblast growth factor (FGF), oxytocin, and vassopressin were purchased from Sigma. Metrizamide was obtained from NycoMed Pharma A/S or Sigma. Platelet-derived growth factor (PDGF; B/B) was purchased from Boehringer. Insulin (human recombinant) was delivered from Novo Nordisk, Denmark.

All stock solutions were prepared in deionized water, except for platelet-derived growth factor, which was prepared in an aqueous stock solution containing 0.1% BSA (w/v). For the experiment testing single amino acids, 30 m*M* stock solutions of each were prepared in a 10 m*M* TRIS/HCl buffer (TRIS) at pH = 7.4 and—if necessary—pH was adjusted to 7.4 with additional TRIS. The control

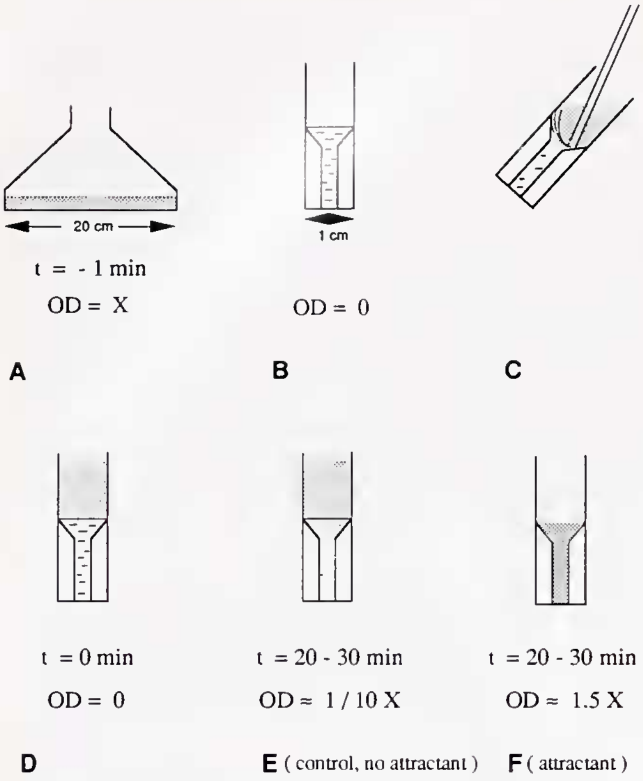

Figure 1. Schematic representation of the quantitative two-phase assay. Cells starved in a 500-ml Fernbach flask at a concentration of 1–2 × 10^5 cells ml^{-1} at a temperature of 21 °C for 20–40 h are used. OD$_{600}$ of the cell suspension was measured just before the experiment, using the solution of metrizamide as reference. In this example, OD of the cell suspension equals X (A). One milliliter of the metrizamide solution is filled into the microcuvettes and each cuvette is blanked (B). Using a pasteur pipette and a steady fingertip, 1.5 ml of the cell suspension is carefully layered on top of the metrizamide (C). Immediately after the cells are applied, the optical density of the lower phase should still be zero (D). After 20–30 min, the OD of the lower phase containing 1 mg proteose peptone ml^{-1} will increase to about 1.5 X, implying that virtually all cells are now present in the lower phase (F). In the control cuvette, the OD is only slightly increased—to about 1/10 X (E).

assays in these experiments contained TRIS at the appropriate concentration.

The mixture of amino acids was prepared in a stock solution composed of 10 m*M* of each of the amino acids found in a synthetic medium proposed for growth of *Tetrahymena* (Rasmussen and Modeweg-Hansen, 1973). These are DL-alanine, L-arginine, L-asparagine, L-glutamate, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tryptophan, and DL-valine. The amino acid mixture was adjusted to pH = 7.4 by the addition of NaOH.

Results

In the experiment shown in Figure 3, cells from late exponential growth phase were starved at a concentration of 1.6 × 10^5 cells ml^{-1}, and assays were performed after 2, 16, 40, and 64 h of starvation using no attractant (controls) or proteose peptone (PP) as attractant in a concentration of 1 mg ml^{-1}. The responses to PP were significant at all four periods of starvation, whereas the background levels (control) exhibited great variation (Fig. 3).

No significant response to amino acids and hormones could be detected after 2 h of starvation. After 16 h of starvation, sensitivity to hormones and amino acids could be observed. However, these responses were often difficult to detect and quantify because of high and unstable background levels in the controls. After 40 h of starvation, the cells were still sensitive to weak attractants and, at the same time, the background levels in the controls became lowered and more stable, thus making the assay more reproducible. In general, it proved to be true that after a certain period of starvation, the background level became low while the sensitivity of the assay was still high. In this (optimal) situation, cells slowly—but continuously—enter the lower phase in the control assay (Fig. 3C). After 64 h of starvation, the sensitivity to weak attractants was markedly decreased, and after 88 h of starvation only response toward PP could be detected. Responses to PP were observed even after 192 h of starvation.

The size of the background level and thus the sensitivity of the assay could be manipulated by the concentration of the metrizamide in the lower phase. This was true only at 40 h of starvation. Attempts to suppress the background levels at 16 h of starvation by increasing the concentration of metrizamide to as much as 20% were not successful. Similarly, increased sensitivity of the assay could not be established by lowering the concentration of metrizamide after 64 h of starvation.

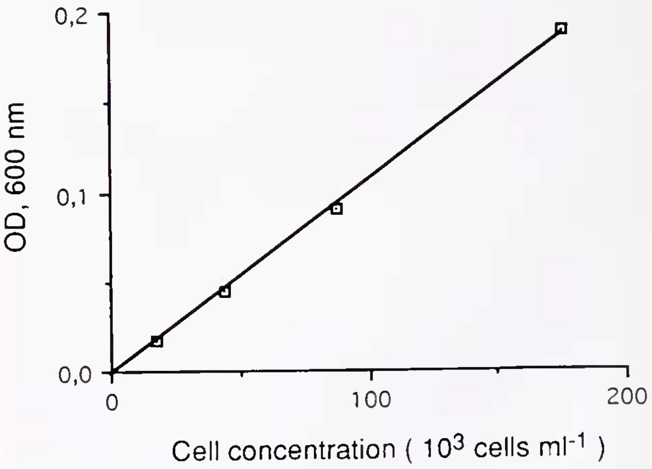

Figure 2. The relationship between OD$_{600}$ and cell number in an aqueous suspension. Careful stirring ensured equal distribution of the cells throughout the suspension.

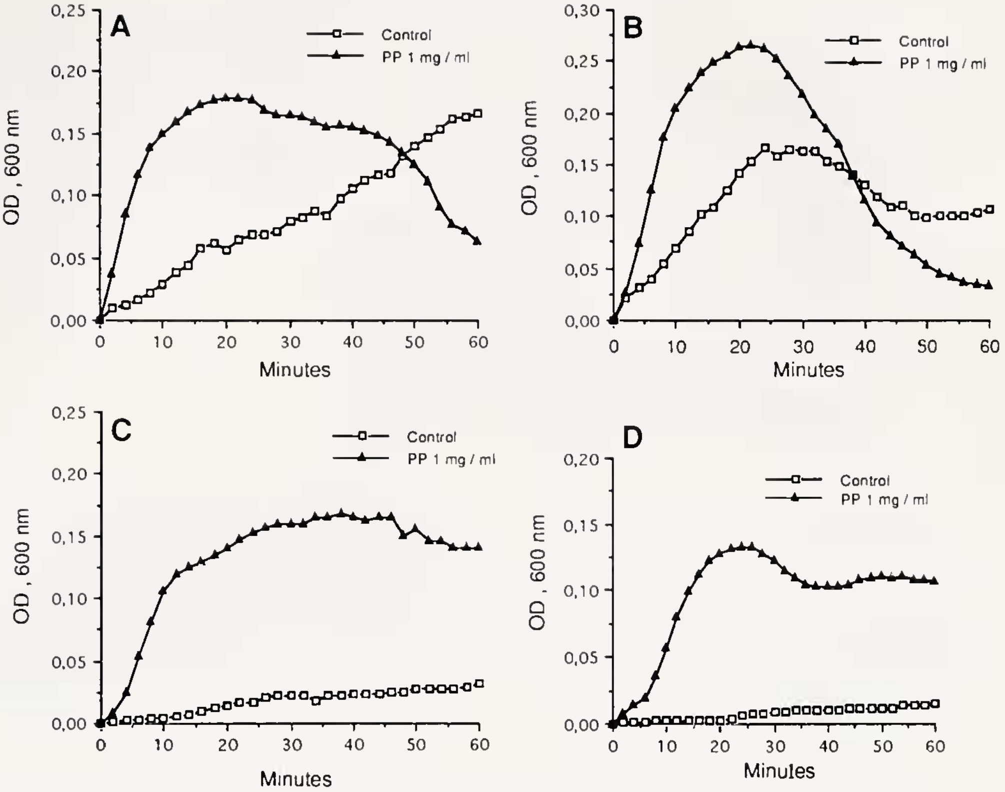

Figure 3. Time-response curves after 2 h (A), 16 h (B), 40 h (C), and 64 h (D) of starvation. The ODs of the cell suspension were 0.19, 0.20, 0.13, and 0.12, respectively, and the maximum ODs in response to PP at a concentration of 1 mg ml^{-1} (▲) were 0.18 (20 min), 0.27 (22 min), 0.17 (38 min), and 0.13 (24 min), respectively. That is, the maximum absolute responses to protease peptone (PP) were 63, 92, 85, and 77%, respectively. The background—control with no attractant (☐)—was drastically reduced after 40 h of starvation in comparison to the background obtained after a shorter period of starvation. The assay was sensitive to weak attractants only between 16 and 64 h of starvation, and the most distinguishable and reproducible quantitative determinations were obtained using cells starved for 40 h.

Cells with maximal sensitivity could be obtained within a shorter period of starvation if cells were grown to the early stationary phase before they were transferred to starvation medium. Thus, the results shown in Figure 4 and Table 1 are from experiments using early stationary phase cells starved for 24 h.

The time-response curves offer three approaches for quantitative analysis: (1) initial slope of the absorbance *versus* time, (2) final maximal absorbance within the time period of measurement, and (3) signal-to-noise ratio (S/N) at a fixed time—*i.e.*, S/N = 1, no chemokinesis; S/N < 1, negative chemokinesis; S/N > 1, positive chemokinesis.

In the experiments shown in Figure 4 and Table 1, we tested a variety of animal peptide hormones, some of which have previously been found to work as chemoattractants for *Tetrahymena*. Table 1 lists the S/N's obtained after 24 min; Figure 4 shows representative time-response curves for a number of attractants tested. Obviously, β-endorphin, FGF, insulin, and PDGF all are good attractants in nanomolar concentrations, whereas oxytocin and vassopressin were not found to be attractants at all. Met-enkephalin induced a significant response, but only in micromolar concentrations; maximum S/N = 3.7 at 200 μ*M*. Acetylcholine was a strong attractant in a way similar to the hormones, but was active only in millimolar concentrations; maximal S/N = 4.1 at 1 m*M* concentration.

PDGF was the most potent attractant of the peptide hormones tested. PDGF was kept in a stock solution of 300 n*M* containing 0.1% BSA. Maximum S/N of PDGF was 5.1 at a concentration of 3 n*M*. At this PDGF concentration, the concentration of BSA was 10 μg ml^{-1}. Although BSA at a concentration of 10 μg ml^{-1}, by itself, did not induce any chemoattraction, the effect of PDGF in the BSA solution might still be due to a synergistic or cooperative effect of the two compounds. However, earlier results obtained with PDGF

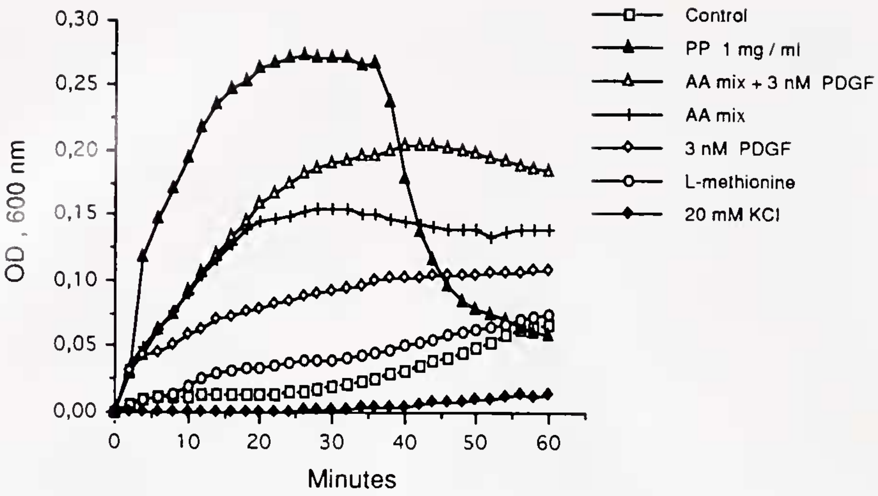

Figure 4. Effect of different attractants and one repellant. OD of the lower phase was monitored every second minute. PP, protease peptone; AA mix, mixture of amino acids (1 m*M* of each); PGDF, platelet-derived growth factor. The plots illustrate the chemosensory responses obtained using PP at a concentration of 1 mg ml^{-1} (▲), AA mix in combination with 3 n*M* PDGF (△), AA mix (+), 3 n*M* PDGF (◇), 5 m*M* L-methionine (○), and 20 m*M* KCl (◆), respectively. OD of the cell suspension used was 0.207. Cuvettes containing no attractant were tested as controls (□). The data shown represent only some of the data in the experiment.

tested in a neutralized HAc-solution seem to indicate that PDGF is itself a very potent chemoattractant to *Tetrahymena* (Hellung-Larsen *et al.*, 1986).

Insulin induced the maximal response at a concentration of 170 n*M*. However, 1.7 *μM* insulin had a repellant effect (S/N = 0.1). Such a dramatic change of effect due to a 10-fold rise in concentration has not been observed for any other peptide attractant. All compounds tested were prepared in stock solution just before the experiment (except for the PDGF solution, which had been stored for approximately 1 year at −20°C). Initial screening revealed that *β*-endorphin had an atypical loss of activity after only a short storage (2–3 days) in aqueous solution at 0°C.

The hormones tested in this experiment were also examined in a capillary assay, and similar results were obtained (results not shown). Furthermore, experiments using the capillary method revealed that *α*-endorphin was also active as an attractant in nanomolar concentrations.

We also tested the 17 amino acids present in Holz-defined medium. The amino acids were screened twice at a concentration of 5 m*M*. Glutamic acid, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine were all positive, with S/N's between 1.5 and 2.5 (results not shown).

We found S/N equal to 9.6 for a mixture of the 17 amino acids (1 m*M* of each amino acid). Furthermore, when PDGF was added to the mixture of amino acids (AA mix) the S/N increased to 11.3. The relatively high molarity of the AA mix did not seem to affect either the viability or the swimming of the cells applied in these assays. This was determined by microscopical analysis of cells taken from both the lower and the upper phases of such assays.

Using S/N values, a hierarchy of chemoattracting potentials of the various compounds can be listed: PP $\gg$ AA mix + PDGF > AA mix > single hormones > single amino acids > control > repellants. A similar order results if the initial slopes of the time-response curves are considered. However, the initial slopes using AA mix or AA mix + PDGF are virtually identical (Fig. 4), indicating that differences can be resolved when measuring S/N values rather than initial slopes. A similar conclusion is drawn when comparing different concentrations of the same attractant. Thus, PP in concentrations of 1, 0.5, and 0.1 mg ml^{-1} gave rise to nearly identical initial slopes, but the maximum responses in these experiments were not similar (Fig. 5).

When a repellant was present in the lower phase, cells were prevented from entering this phase. We found that 20 m*M* KCl, 1 m*M* 4-nitroaniline, and 100 *μM* 2,4-dinitroaniline each almost completely prevented the cells from migrating into the lower phase. This was true even if PP (in any concentration) was present in the lower phase in combination with the repellant (results not shown). The sensitivity of the assay to repellents is independent of the starvation period of the cells. That is, any (detectable) background level in the assay will be suppressed when a repellant is present therein.

Table I

Chemoattractant activity of peptides and amino acids in the two-phase assay

Compound	Conc. (w/v)	Molarity	S/N at 24 min after setup
Control (no attractant)	—	—	1.0 ± 0.3 (10)
Platelet-derived growth factor	1 μg/ml (BSA 100 μg/ml)	30 nM	1.4
	100 ng/ml (BSA 10 μg/ml)	3 nM	5.1 ± 0.6 (4)
	10 ng/ml (BSA 1 μg/ml)	300 pM	2.1
Bovine serum albumin	100 μg/ml	—	1.3
	10 μg/ml	—	1.0
	1 μg/ml	—	1.2
Insulin	10 μg/ml	1.7 μM	0.1
	1 μg/ml	170 nM	4.7 ± 0.8 (4)
	100 ng/ml	17 nM	2.3
Fibroblast growth factor	1 μg/ml	75 nM	4.0 ± 0.4 (10)
	100 ng/ml	750 pM	1.0
β-endorphin	250 ng/ml	70 nM	1.8
	25 ng/ml	7 nM	3.0 ± 0.4 (4)
	2.5 ng/ml	700 pM	1.6
Met-enkephalin	100 μg/ml	200 μM	3.7 ± 0.5 (4)
	10 μg/ml	20 μM	2.1
	1 μg/ml	2 μM	1.0
Acetylcholine	1.8 mg/ml	10 mM	3.7
	180 μg/ml	1 mM	4.1 ± 0.6 (4)
	18 μg/ml	100 μM	1.1
Oxytocin	10 μg/ml	10 μM	0.8
	1 μg/ml	1 μM	0.8
	100 ng/ml	100 nM	1.0
	10 ng/ml	10 nM	1.0
	1 ng/ml	1 nM	1.0
Vassopressin	10 μg/ml	9 μM	0.8
	1 μg/ml	900 nM	1.0
	100 ng/ml	90 nM	1.0
	10 ng/ml	9 nM	0.9
	1 ng/ml	900 μM	1.0
Amino acid mixture (AA mix)	(5.0 mM of each)	115 mM	7.8
	(1.0 mM of each)	23 mM	9.6 ± 0.5 (4)
	(200 μM of each)	460 μM	5.1
AA mix +			
Platelet-derived growth factor	(1.0 mM of each) + 100 ng/ml (BSA 10 μg/ml)	23 mM + 3 nM	11.3 ± 0.7 (4)
Proteose peptone	1 mg/ml	—	17.5 ± 1.4 (4)

Signal/noise ratios (S/N) at 24 min of assaying are listed. Some determinations were performed in quadruplicate and are given as mean values of S/N ± standard deviation. All results presented originate from the same culture of starved cells. That is, all assays were performed within 6 h. Corresponding time-response curves of the experiment are shown in Figure 4. BSA = bovine serum albumin.

As previously stated, all experiment were performed at 28°C (Leick *et al.*, 1990). However, we reinvestigated the effect of temperature on the chemokinesis of *Tetrahymena* by performing experiments at 15°, 25°, and 35°C. Parallel assays were performed in three spectrophotometers with thermostats set to 15°, 25°, and 35°C, respectively. The responses were strongest at 25°C, delayed at 15°C, and markedly reduced at 35°C (Fig. 6). This was true for any concentration of PP, thus confirming that the optimal temperature for chemokinesis in *Tetrahymena thermophila* is about 25°C.

Discussion

In this paper, we have described a number of parameters for carying out the two-phase assay for chemokinesis in *Tetrahymena*. The sensitivity of the assay depends upon the period of starvation. Maximum reproducibility and sensitivity is reached within 20–40 h of starvation. It seems that during starvation the cells gradually lose their ability to pass the water-metrizamide interphase unless stimulated with chemoattractant. The reason for this phenomenon is not known. The results may suggest that *Tetra-*

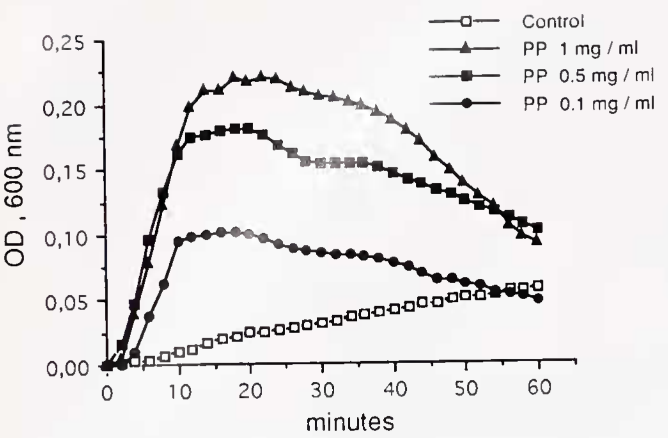

Figure 5. Effect of proteose peptone (PP) in different concentrations. PP was tested in final concentration of 0.1 mg ml^{-1} (●), 0.5 mg ml^{-1} (■), and 1 mg ml^{-1} (▲). Cuvettes containing no attractant were tested as controls (□).

hymena, during starvation, undergoes a progressive decrease in cellular density, which in turn may affect the ability of the cells to enter the metrizamide phase. The metrizamide phase itself is known to reduce the swimming speed of the cells slightly (Hellung-Larsen *et al.*, 1986). The progressive decrease in swimming speed and cell volume known to take place during starvation might also offer some explanation of the observed phenomenon (Hellung-Larsen *et al.*, 1993).

During continued starvation, sensitivity to most attractants is gradually lost according to the relative strengths of the chemoattractants. It is remarkable, however, that sensitivity to PP was never lost. In addition to being a chemoattractant, PP is known to increase the swimming speed of the cells (Hellung-Larsen *et al.*, 1986). The stimulation by PP of the swimming speed may in part explain the strong chemosensory responses induced by this chemoattractant.

The results confirm that amino acids and peptides are chemoattractants for *Tetrahymena*. The responses to a mixture of amino acids were strong in comparison to the responses obtained using single amino acids. This additive effect may indicate a mechanism in which the amino acids work through specific receptors. Specific receptors may also explain the additive effect seen when AA mix + PDGF are tested in the assay.

We have confirmed the chemoattractive effect of PDGF, FGF, β-endorphin, and acetylcholine shown earlier (Tsang and Levandowsky, 1983; Andersen *et al.*, 1984; Leick and Hellung-Larsen, 1985; Hellung-Larsen *et al.*, 1986; O'Neill *et al.*, 1988). Furthermore, we have demonstrated a similar effect of insulin.

The fine adjustment of the starvation period necessary for optimal sensitivity towards attractants is not needed when testing repellants. Therefore, the assay might also be useful as a standard screening procedure in aquatic toxicology, because *Tetrahymena* is repelled from industrial pollutants such as anilines, phenols, and naphthalenes (Berk *et al.*, 1990; Pauli and Berger, 1992). Screening for repulsive effect of pollutants could be carried out in the two-phase assay as a competition assay with PP as the attractant.

The two-phase assay, in its present form, provides a sensitive and easy-to-perform spectrophotometric assay for chemokinesis in *Tetrahymena.* We infer that the assay could also be useful for testing chemokinesis in other ciliates and perhaps some flagellates. Such an approach will

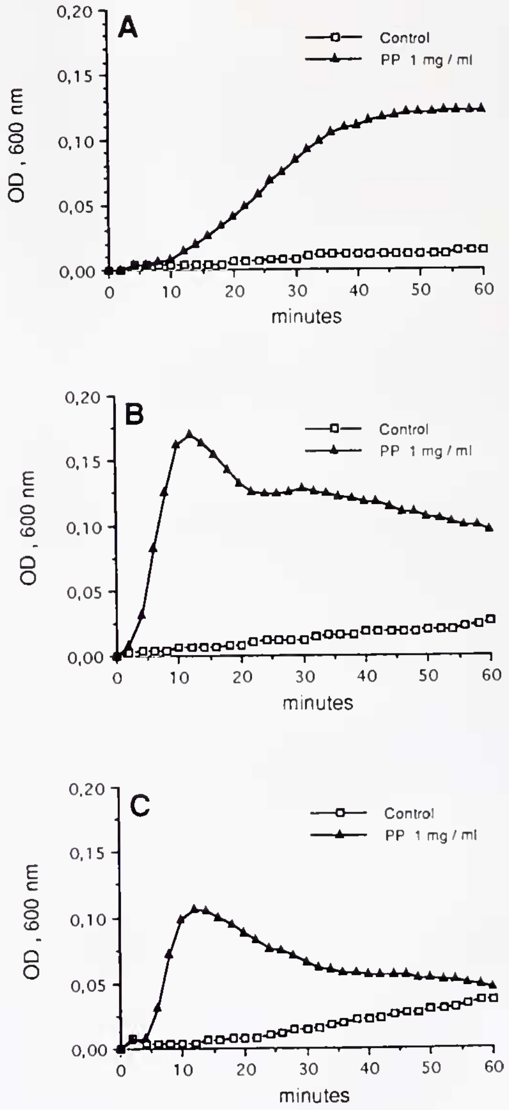

Figure 6. Effect of temperature on the chemosensory behavior of *Tetrahymena thermophila* Parallel assays were performed at 15°C (A), 25°C (B), and 35°C (C) using no attractant (□) or protease peptone (PP) at a concentration of 1 mg ml^{-1} in the assay (▲). OD of the cell suspension used was 0.15.

need proper adjustment of the metrizamide concentration in the lower phase and of the conditions for the starvation of the organism being tested.

Acknowledgments

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